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Sufficient intracellular survival capability is essential for induction of protective immune response by the *Brucella* live attenuated vaccine M5-90

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The fact that live attenuated vaccine but not killed Brucella cells could induce protective immune response against Brucellosis implied that survival capability in host is important for the immune response induction. Member of the Omp25 protein family are shown to be important for *Brucella* virulence and immunological regulation. Omp25 mutants of virulent strains shows reduced virulence and could provide protection against virulent strain challenge, indicating that Omp25 is important for intracellular survival but dispensable for immunological protection. In the present study, to further define roles of Omp25 and clarify the possible protection mechanism of live vaccine strains, mutant strain M5Δomp25 of a live attenuated strain M5 was constructed and analyzed. The mutant was rapidly cleared from macrophage and mice when compared with wild type strain, indicating their reduced intracellular survival and virulence. The mutant induced decreased antibody responses and elicited lower level of IFN-γ and IL-10 in mice than their parent strain M5, implying reduced both humoral and cellular immunity induced by the mutant. Protection efficiency assay showed that M5Δomp25 could provide protection against M5 challenge but not the virulent strain 16M challenge. This indicated that sufficient survival capability is essential for induction of protective immune response for the live attenuated vaccine M5. This finding is helpful for screening and development of live attenuated or genetically marked vaccines.

Key words: *Brucella*, Omp25, intracellular survival, protective immune response.

INTRODUCTION

Bacteria of the genus *Brucella* are Gram-negative facultative intracellular pathogens that cause severe zoonotic disease (Young, 1983, Boschiroli et al., 2001). Live attenuated vaccines are shown to be the most efficient vaccine form for prevention of both human and animal brucellosis (Garin-Bastuji et al., 1998, Schurig et al., 2002). Although with great protection provided, live attenuated vaccine have some limitations, including the high antibody response that interferes with sera-diagnosis, unknown genetic background, and possibility reversion into virulent ones (Jimenez de Bagues et al., 1992, Blasco, 1997).

Therefore, great attempts are being made to develop genetically marked vaccines based on rational design to

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overcome these limitations. For example, inactivation of *vjb*R not only attenuates the strain, but also provides a choice for differentiation of vaccine strains from virulence strain by serological methods (Wang et al., 2011b). On the other hand, the fact that live attenuated vaccine but not the killed *Brucella* cells could provide efficient protection against challenges implied that survival capability in host might be important for induction of protective immune response.

For development of genetically marked live attenuated vaccine, deletion of an antigen in the strain could provide a choice for differentiation of vaccine strain and virulence strains. Therefore, the choice of immunogenic protein is important for screening and development of this type of vaccines. Outer membrane proteins (OMPs) are important antigens in many pathogenic bacteria because of their easy recognition by host immune system. OMPs are important for maintaining the integrity and selective permeability of bacterial membranes, and play important roles in bacterial pathogenesis by enhancing the adaptability to various environments (Cloeckaert et al., 2002). Outer membrane proteins are differentially expressed when intracellular survival related gene is inactivated, indicating their roles in virulence (Wang et al., 2009; Uzureau et al., 2007; Guzman-Verri et al., 2002). The OMPs of *Brucella* spp. were initially identified in the early 1980s. At present, three groups of OMPs were identified and classified according to their apparent molecular masses: group 1 (31 to 34 kDa), group 2 (36 to 38 kDa) and group 3 (25 to 27 kDa) (Verstreate et al., 1982; Edmonds et al., 2002a). The group 3 OMPs is also termed Omp25/Omp31 family and consists of seven homologous OMPs: Omp25, Omp25b, Omp25c, Omp25d, Omp31, Omp31b and Omp22 (Salhi et al., 2003; Vizcaino et al., 2004).

Omp25 is linked to the peptidoglycan layer by a covalent bond. Association of Omp25 with lipopolysaccharide (LPS) is important for the maintenance of conformational epitopes of this protein (Cloeckaert et al., 1996). Besides being a major component in the *Brucella* outer membrane, Omp25 is also antigenic in both mice and cattle. Vaccination of mice with either *Brucella* cell envelope extracts containing high levels of Omp25 or native Omp25 in an oil-based adjuvant provides significant protection against virulent challenge (Winter and Rowe, 1988; Montaraz and Winter, 1986). Mutant *Brucella melitensis*, *Brucella abortus*, and *Brucella ovis* strains with the *omp25* gene inactivated have been found to be attenuated in mice, goats (*B. melitensis*) and cattle (*B. abortus*) (Edmonds et al., 2001; Edmonds et al., 2002a). Furthermore, vaccination of mice with *B. melitensis* 16M *omp25* mutant afforded protection against virulent strain 16M with efficiency equivalent to the current vaccine strain Rev.1, indicating Omp25 do not play essential roles in eliciting immunological protection in *B. melitensis* virulent strain (Garin-Bastuji et al., 1998; Edmonds et al., 2002a; Edmonds et al., 2002b). These data indicated that Omp25 is appropriate for development of genetically marked attenuated vaccine strains from virulence strains.

To further test the possibility of use of Omp25 for development of genetically marked vaccine from vaccine strains, and also roles of the survival capability of attenuated vaccine in immune protection, a mutant strain were constructed from a live attenuated strain M5. The intracellular survival, antibody response, cell mediated immunity and protection efficiency was analyzed and compared.

MATERIALS AND METHODS

Ethics statement

All animals were handled in strict accordance with good animal practice as defined by the relevant national animal welfare bodies, and the animal work was approved by Beijing Institute of Disease Control and Prevention animal ethics committee (Ethical Approval BIDCP001-2008).

Bacterial strains and primers

The *Brucella* strain M5-90 (M5) is a live vaccine strain licensed and extensively applied in China (Wang et al., 2011a). This strain was developed in 1962 by the Haerbin Institute of Veterinary Medicine at the Academy of Agriculture Sciences. The vaccine is derived from the virulent strain of *B. melitensis* M28 and used mainly to vaccinate both pregnant and non-pregnant sheep (Chen et al., 1990; Deqiu et al., 2002). Virulent strain 16M was used for challenge. *Brucella* was cultured in Tryptic Soy Broth (TSB) or Tryptic Soy Agar (TSA). *E. coli* strain DH5α was grown on Luria-Bertani (LB) medium. Plasmid pBBR1MCS-2, a broad host range plasmid capable of replicating in *Brucella,* was kindly provided by Professor Kenneth M. Peterson. Primers and their sequences used in this study are listed in Table 1.

Construction and confirmation of M5Δomp25 mutant

To obtain deletion mutants, a new plasmid pUC19K was firstly constructed. A pair of primers pUC19K-F and pUC19K-R were synthesized, with restriction enzyme sites of *Bam*HI, *Xho*I and *Apa*I added to the 5′ end of pUC19K-F and *Eco*RV, *Xba*I, *Mlu*I and *Sal*I to the 5′ end of pUC19K-R. The kanamycin gene was amplified from pBBR1MCS-2 with primer pUC19K-F and pUC19K-R and cloned into suicide plasmid pUC19, generating pUC19K.

The deletion mutant M5Δomp25 was constructed with pUC19K as follows. The upstream homologous arm omp25-N of *omp25* was amplified with primers omp25-N-F and omp25-N-R, and the downstream arm omp25-C with omp25-C-F and omp25-C-R (Table 1). The two homologous arms were sequentially cloned into the two multi-cloning sites (MCS) of pUC19K (with omp25-N cloned at *Kpn*I - *Xho*I sites and omp25-C at *Sal*I - *Hind*III sites) to generate suicide plasmid pUC19K-omp25NC containing a disrupted *omp25* gene. Competent M5 was electroporated with pUC19K-omp25NC and the *omp25* deletion mutant M5Δomp25 was isolated by its amp^S kan^R phenotype. The deletion mutant was further confirmed by PCR amplification with primer puc19k-F and omp25-I-R, located in kamamycin gene and downstream of homologous arm of omp25 respectively. PCR products were sequenced to confirm the sequence.

To confirm the dysfunction of *omp25*, M5Δomp25 was cultured in TSB, and transcription of omp25 was analyzed by RT-PCR as described previously (Wang et al., 2009).

Table 1. Primers used in this study.

Primer	Sequence（5'→3'）
pUC19K-F	ACGTGGATCCCTCGAGGGGCCCGCCACCTGGGATGAATGTC
pUC19K-R	ACGTGTCGACTCTAGAGATATCACGCGTCGGTCATTTCGAACCCCAGA
omp25-N-F	ACGTGGTACCTGATGCGAGATGCAATGAC
omp25-N-R	ACGTCTCGAGGGAGGCTGTTCCTGGATGG
omp25-C-F	ACGTGTCGACTGATCTGGCCGGTACGACTG
omp25-C-R	ACGTAAGCTTAAATTCATCAACCCTGGCTC
omp25c-N-F	ACGTGGTACCGTTTCAAGTTCCTGCCCACG
omp25c-N-R	ACGTCTCGAGGCCGAGATAGGCACCATTCC
omp25c-C-F	ACGTGTCGACGACGACGCTGCCGTTACCA
omp25c-C-R	ACGTAAGCTTCCTGACCGCCGAAATAAGC
omp25-I-R	ATGCGGAACGCTATGAAG
omp25c-I-R	GTTGTAGCCGGTGTAAAGG

Sensitivity to polymixin and stresses

The susceptibilities of *Brucella* strains to polymyxin B (Sigma) were determined following a protocol described previously with some modifications (Martinez de Tejada et al., 1995). *B. melitensis* strains were cultured on TSA for 72 h. Then, bacterial suspensions of approximately 1×10^4 CFU/ml were prepared in PBS and 100 µl aliquots were mixed with 100 µl polymyxin B of different concentrations (final concentrations in the wells were 2,000, 1,000, 500 and 250 µg/ml, respectively) and cultured in 96-well plates. After 1 h of incubation at 37°C in a 5% CO_2 atmosphere, 50 µl aliquot of each well was serially diluted and spread in triplicate on TSA plates for CFU determination. The survival percent was calculated by dividing the CFU of survived bacteria by that of the inoculated ones. The results were expressed as the mean ± SD of three assays. All the results represent the averages from at least three separate experiments.

The sensitivity of *Brucella* strains to hydrogen peroxide, high salinity or high osmolarity stress were determined as follows. *B. melitensis* strains inoculated into TSB medium were grown to the early logarithm phase (OD_{600} = 0.6) at 37°C. To determine the effect of high salinity or high osmolarity stress on *B. melitensis*, the log-phase cells were incubated at 37°C for 20 min in the presence of NaCl (1.5 mol/L) or sorbitol (1.5 mol/L). To test the effect of oxidative stress, the cells were incubated for 30 min in 440 mmol/L H_2O_2. After the treatment, the survival percent of the bacteria was determined as above.

Survival experiments

For intracellular survival assay, murine macrophage-like J774A.1 was infected with M5 and M5Δomp25, and survival capability was analyzed essentially as described previously (Zhong et al., 2009). The assays were performed in triplicate and repeated once.

Survival of *Brucella* in mice was assayed as follows. 6-week-old female BALB/c mice were inoculated intra-peritoneally (i.p.) with a total of 1×10^7 CFU of deletion mutants or M5. Survival or persistence of the bacteria in mice was evaluated by enumerating the bacteria in the spleens at different time points post the infection. At 3, 7, 14, 28 days post the inoculation, mice were euthanized and spleens were removed aseptically. The spleens were homogenized in 1 ml PBS and serially diluted, followed by plating on TSA plates. Recovered bacteria were enumerated to evaluate the survival capability in mice.

Immunization and antibody surveillance

Female 6-week-old BALB/c mice were randomly divided into groups and inoculated i.p. with 200 µl PBS containing 2×10^5 CFU of M5M5Δomp25, or 200 µl PBS. To evaluate antibody responses, serum samples were obtained from immunized mice at 7, 14, 28, 42, 56 days post the immunization (p.i.) and IgG was determined by ELISA.

Cytokine production assay

At 7, 14, 28, 42 56 days p.i., five mice from each group were sacrificed and their spleens were aseptically recovered. Single cell suspensions from the spleens were obtained by homogenization. After washing three times with PBS, the cell suspension was re-suspended in complete RPMI 1640 medium (GIBCO BRL) supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 2 mM L-glutamine. The cell were cultured in 96-well plates for 72 h at a concentration of 5×10^5 cells/well in the presence of heat-killed M5 at 1×10^8 CFU/well, 0.5 µg of concanavalin A (ConA, positive control) or medium alone (negative control), respectively. Then the plates were centrifuged at 1000 rpm for 10 min, the clear culture supernatants were collected and stored at -20°C. IFN-γ and IL-10 were determined by using an ELISA Quantikine Mouse kit (R&D Systems).

Protection efficiency evaluation

56 day p.i., mice were challenged i.p. with 1×10^5 CFU per mouse (200 µl) of virulent strain 16M. At 7, 14, 28 days post the challenge, mice were killed and the spleens were removed aseptically. Bacterial cell number in each spleen was determined as described above.

Statistical analysis

Bacteria survival in macrophage and in mice was expressed as the mean $\log_{10}$ CFU ± the standard deviation (SD). Antibody response was expressed as the mean OD_{450} ± SD. Cytokine production *in vitro* was expressed as mean cytokine concentration ± SD. The protective efficiency at different time points was expressed as the mean $\log_{10}$ CFU ± SD. The differences between groups were analyzed by ANOVA followed by Tukey's honestly significant difference post test comparing all groups with one another. For ANOVA, P values of < 0.05 were considered statistically significant.

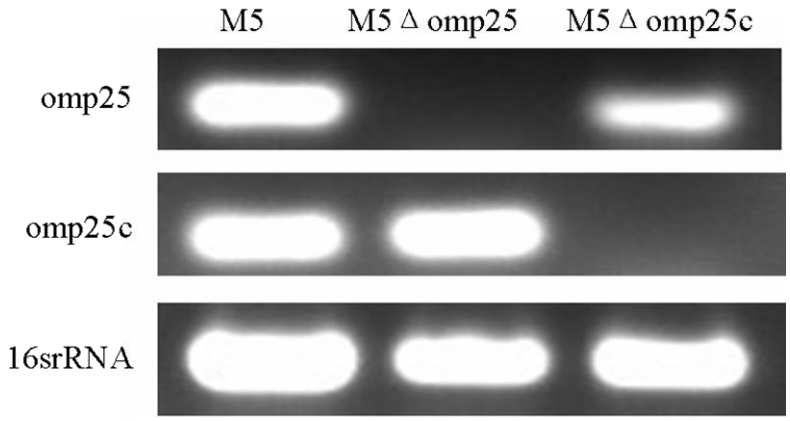

Figure 1. Transcriptional confirmation of the *omp25* mutant. Transcription of *omp*25 was verified by RT-PCR; transcripts of *omp*25 was detected in M5, but not in M5Δomp25.

RESULTS

Construction and confirmation of deletion mutants of *omp25*

To construct resistance gene replacement mutant of *omp25*, a new suicide plasmid pUC19K was constructed by insertion of a kanamycin gene at multi-cloning site of pUC19. Enzyme digestion and sequence analysis showed that the recombinant plasmid pUC19K was correctly constructed (data not shown). Then, this plasmid was used to construct deletion mutant of *omp25* as described above. The deletion mutants were selected by isolating kan^R and amp^S colonies. PCR verification and DNA sequencing showed that *omp25* was successfully replaced by kanamycin gene in M5Δomp25 (data not shown). To further confirm the mutant, transcriptions of *omp25* gene was quantified by RT-PCR. As shown in Figure 1, the transcription of *omp25* was detected in M5, but not in M5Δomp25, indicating the *omp25* was successfully inactivated in M5Δomp25.

M5Δomp25 showed increased sensitivity to polymixin B and *in vitro* stresses

Outer membrane structure plays important roles in *Brucella* adaptation to stress environments. To evaluate the effect of the inactivation of *omp25* on *B. melitensis* M5 outer membrane (OM) property, we first tested viability of M5Δomp25 after controlled exposure to polymyxin B. when compared with M5, *omp25* mutant showed no increase in sensitivity to polymyxin B until the concentration was increased to 4 mg/ml (Figure 2A). This implied that *omp25* does not contribute greatly to outer membrane's resistance to polymyxin B. Then, we compared survival capability of M5Δomp25 and M5 under stresses of hydrogen peroxide, high salt and high osmosis that simulate intracellular hostile environments. As shown in Figure 2B, in standard culture of TSB, the two strains showed identical survival capability. However, when the strains were subjected to the stresses, the survival percents of M5Δomp25 was greatly lower than that of M5, implying that Omp25 is important for *Brucella* resistance to these stresses.

Reduced survival of M5Δomp25 in macrophages and mice

Intracellular survival is a key characteristic phenotype of *Brucella*. To evaluate the role of Omp25 in intracellular survival of M5, J774A.1 macrophages were infected with M5Δomp25 and their parent strain M5 at a multiplicity of infection (MOI) of 200. At different time point post infection, intracellular bacteria were isolated and enumerated. From 0 to 4 h post infection, no significant difference ($P > 0.05$) was observed between M5 and the mutant (Figure 3A). Intracellular bacteria of the two strains decreased from 10^7 to 10^5. But at later time points, although the number of strains decreased progressively, a more sharp decrease in cell number of M5Δomp25 than that of M5 ($P < 0.05$) was observed from 12 to 24 h p.i. These implied that Omp25 is not important for early invasion of the live attenuated strain, but are important for subsequent intracellular survival in macrophage.

To further evaluate the role of Omp25 in the virulence, BALB/c mice were inoculated i.p. with a high dose (1×10^7 CFU) of M5Δomp25 and M5, respectively, and then the number of bacteria in spleen of infected mice was determined at selected intervals post the infection. The three strains showed similar kinetics of survival in mice (Figure 3B). At 3 d p.i., approximately 10^5 CFU of the mutant and M5 were detected, no differences were observed among the strains, implying that Omp25 are not important for acute infection of *Brucella*. But after that time point, the number of *Brucella* in spleens increased and peaked at 7 d p.i. and then decreased progressively. The CFU/spleen of mice infected with M5Δomp25 was signi-

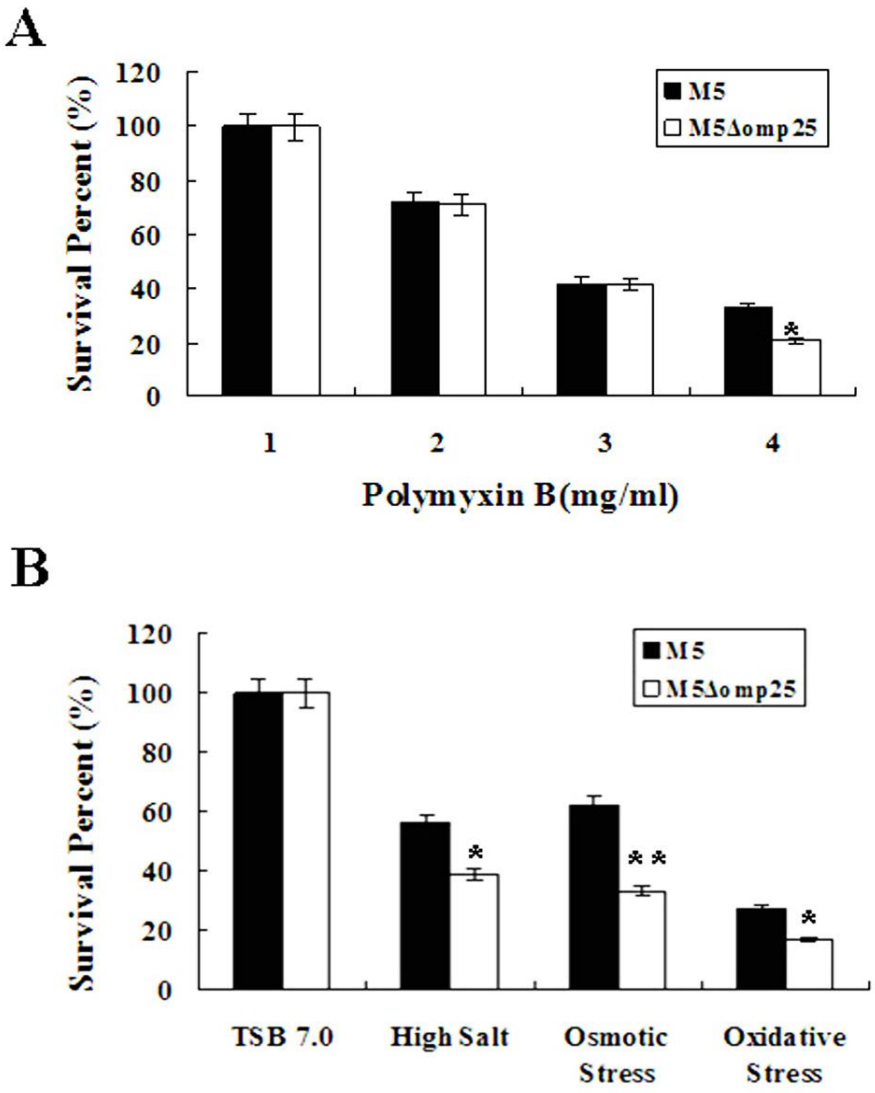

Figure 2. Sensitivity to polymixin B and *in vitro* stresses. (A) Survival percents of M5 and M5Δomp25 after controlled exposure to polymixin B. The two strains were subjected to different concentrations of polymixin B and then survival percent was calculated. M5Δomp25 showed lower survival level than M5 under 4 mg/ml of polymixin B. (B) Increased sensitivity of M5 and M5Δomp25 to *in vitro* stresses. The two strains were subjected to high salt, osmotic and oxidative stresses and the survival percents were calculated. **P*< 0.05; ***P*< 0.01.

ficantly lower than that of M5 from 7 to 28 d p.i.

M5Δomp25 induced lower antibody response in mice

To evaluate the influence of Omp25 on humoral immunity of M5, sera from mice inoculated with M5Δomp25 and M5 were assayed for the presence of *Brucella*-specific antibody by ELISA at selected intervals post infection. As shown in Figure 4, antibodies were detected in sera of mice immunized with M5 or M5Δomp25, but not in that immunized with PBS, indicating the two strains could induce *Brucella*-specific antibodies. However, when compared with M5, M5Δomp25 induced decreased levels of antibodies.

In mice immunized with M5, the IgG levels peaked at 28 PID (OD_{450} = 0.84) and titers remained at a high level at 42 and 56 PID (OD_{450} = 0.64 and OD_{450} = 0.67, respectively), whereas in mice inoculated with M5Δomp25, the IgG levels remained relatively stable ($P > 0.05$) and the OD_{450} was lower than 0.27 at all time points tested. These data indicated a significant difference in IgG levels between the mice inoculated with M5 and those inoculated with M5Δomp25 that occurred, and the deletion of *omp25*

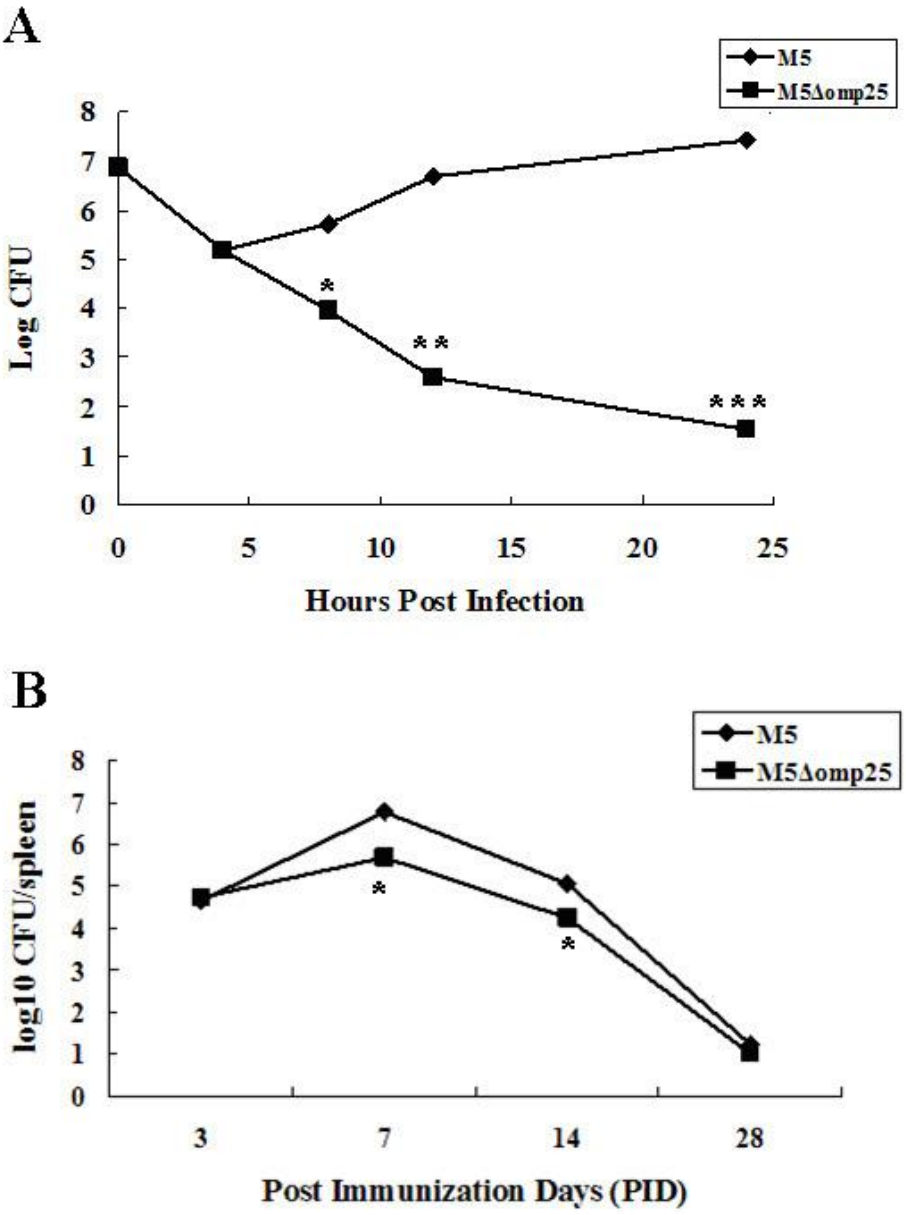

Figure 3. Survival of M5Δomp25 in macrophages and BALB/c mice. (A) Survival of M5 and M5Δomp25 in J774A.1 macrophage. J774A.1 macrophage was infected with M5 and M5Δomp25 and then intracellular bacterial were enumerated. M5Δomp25 showed decreased survival capability in macrophage. (B) Survival of M5 and M5Δomp25 in mice. BALB/c mice were infected with M5 or M5Δomp25; bacteria were isolated and enumerated at different time post infection. **P*< 0.05; **, *P*< 0.01; ****P*< 0.001.

negatively affect the antibody response elicited by M5.

Decreased production of IFN-γ and IL-10 in splenocytes of M5Δomp25 immunized mice

Splenocytes were isolated from inoculated mice at different time point post immunization and stimulated as described above, and IFN-γ and IL-10 were detected in supernatant of the cell culture. As shown in Figure 5A, both M5 and M5Δomp25 were able to elicit the production of IFN-γ. In general, when stimulated with heat-inactivated *Brucella meletensis*, the splenocytes from mice inoculated with M5 produced significantly higher level of IFN-γ than those from mice inoculated with M5Δomp25 ($P < 0.0001$). The production of IFN-γ was observed in splenocytes of M5 immunized mice early at 7 PID, and then increased to the highest level (2441 pg/ml) at 42 PID and remained at

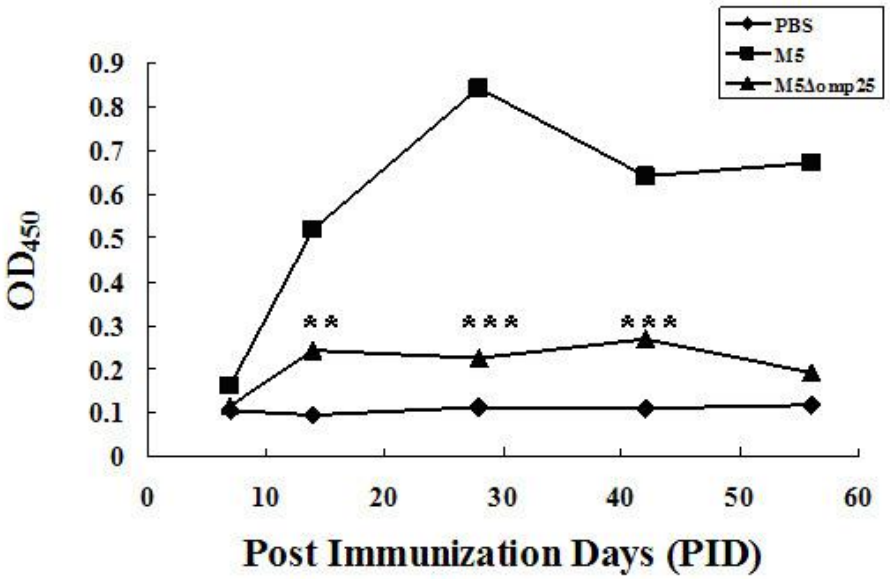

Figure 4. Antibody responses to M5 and M5Δomp25 in immunized BALB/c mice. BALB/c mice were inoculated intraperitoneally with M5, M5Δomp25 or PBS. At 7, 14, 28, 42 and 56 PID, sera were collected and assayed by ELISA for IgG titers. ***P*< 0.01; ****P*< 0.001.

this high level at 56 PID. IFN-γ was not detected in splenocytes of M5Δomp25 immunized mice at 7, 14 and 28 PID and only a low level of it was detected at 42 and 56 PID. The late appearance and transient increase of IFN-γ production by splenocytes of M5Δomp25 immunized mice suggested the important roles of Omp25 in inducing cell mediated immunity.

We then analyzed IL-10 production by heat-inactivated *B. melitensis* stimulated splenocytes. As shown in Figure 5B, the IL-10 production profiles were different from those of IFN-γ. For both groups, IL-10 was detected early at 7 PID and peaked at 42 PID, no significant difference in IL-10 production between the M5Δomp25 and M5 immunized mice was observed ($P > 0.05$) at 7, 14, 28 PID. However, a significant difference was detected at 42 and 56 PID ($P < 0.001$). Levels of IL-10 production from the M5Δomp25 immunized mice were significantly lower than that of M5 immunized ones at 42 and 56 PID.

M5Δomp25 could not confer protection against 16M challenge

The above experiments showed that the intracellular survival and induction of both humoral and cell mediated immunity of M5Δomp25 were greatly reduced, implying possible decreased protection. To evaluate the role of Omp25 in the protection efficiency of M5, mice immunized with M5, M5Δomp25 and PBS were challenged with virulent strain of 16M. At 42 d p.i., no *Brucella* was isolated, indicating that the vaccine strains were completely cleared (data not shown). Then, the immunized mice were challenged with 1×10^5 cfu of 16M at 56 PID. At 7, 14 and 28 days post challenge, *Brucella* in spleen were isolated and enumerated. As shown in Figure 6, the wild type vaccine strain M5 exhibited a significant degree of protection ($P < 0.01$) against 16M when compared with PBS control at 7, 14 and 28 days post the challenge. However, M5Δomp25 did not show significant reduction in spleenic bacterial load in comparison with PBS control ($P > 0.05$). These results indicated that the lack of Omp25 in vaccine strain M5 could not confer protection against virulent *Brucella* challenge.

DISCUSSION

Live attenuated vaccine is the most efficient vaccine form for Brucellosis. The most widely used vaccines include *B. abortus* S19, *B. melitensis* Rev.1, and the recently licensed *B. abortus* RB51. *B. melitensis* M5-90 is originated from a virulence *B. melitensis* strain M28 isolated from sheep by serial passages in chickens (Wang et al., 2011a). This vaccine strain is extensively used for vaccination in China for prevention of animal Brucellosis. In this study, we evaluated the survival capability and induction of immune response and their relation with protection by analyzing a mutant strain M5Δomp25 derived from M5-90.

Outer membrane proteins play important roles in pathogenic bacteria. In addition to be protective antigen candidates, they contribute to membrane integrity which are essential for survival under different environments (Moriyon and Lopez-Goni, 1998). Omp25, a member of the Omp25/Omp31 family, is a major component of *Brucella* outer membrane and highly conserved in *Brucella*

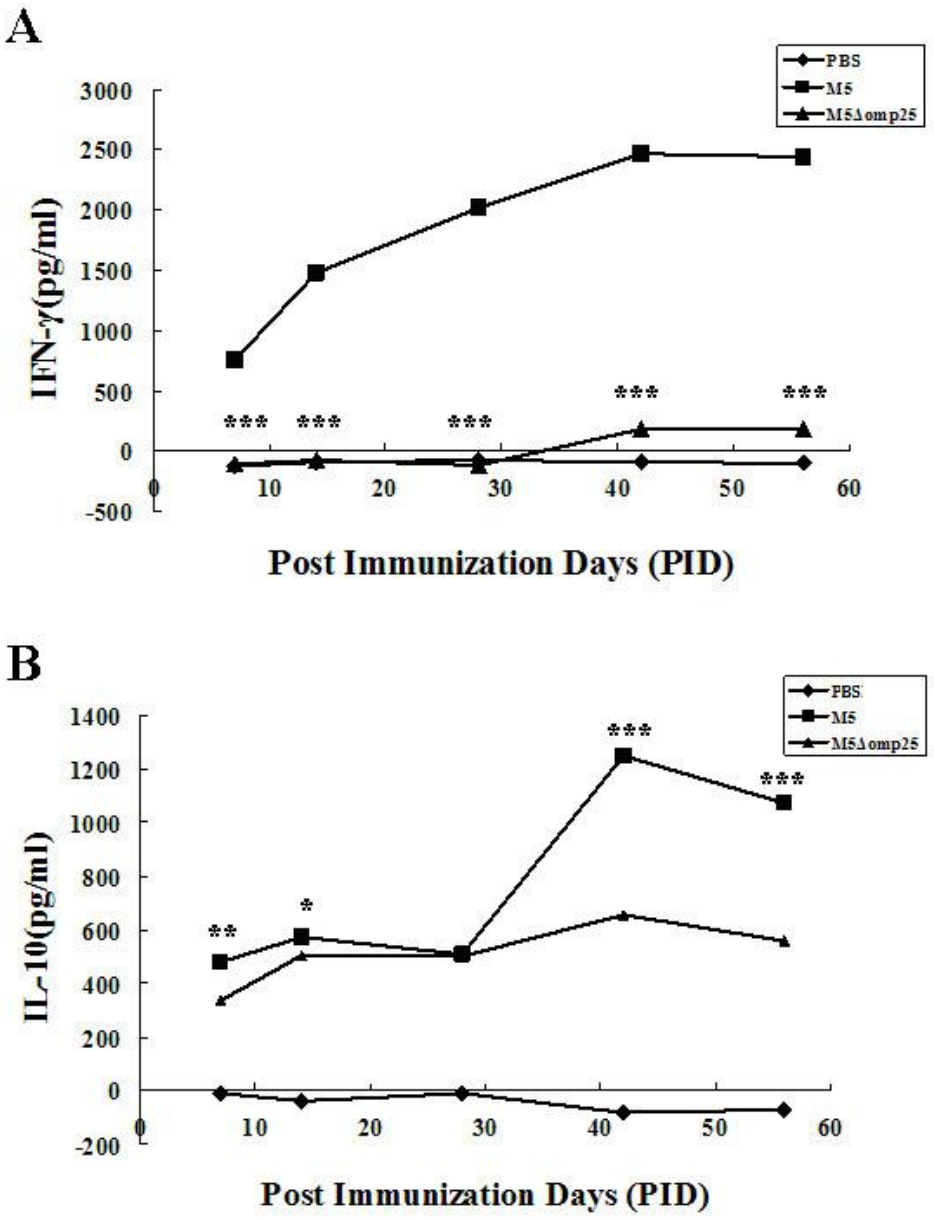

Figure 5. IFN-γ and IL-10 productions by splenocytes of M5 and M5Δomp25 immunized mice. BALB/c mice were immunized with M5, M5Δomp25 or PBS. At different time points after the immunization, splenocytes were isolated and stimulated with either ConA, 1×10^8 CFU heat-inactivated *B. melitensis* M5, or RPMI 1640. The supernatants were collected and IFN-γ and IL-10 productions were assayed by ELISA. (A) Secretion of IFN-γ by splenocytes of M5Δomp25 immunized mice (B) IL-10 production by splenocytes of M5Δomp25 and M5 infected mice. **P*< 0.05; ***P*< 0.01; ****P*< 0.001.

species (Martin-Martin et al., 2008). Omp25 deletion mutants of *B. melitensis*, *B. abortus* and *B. ovis* are attenuated in mice, indicating that Omp25 is involved in the pathogenesis of *Brucella* virulent strains (Edmonds et al., 2001; Edmonds et al., 2002a; Caro-Hernandez et al., 2007).

When compared with M5, M5Δomp25 exhibited altered outer membrane properties, as indicated by its resistances to polymyxin B, hydrogen peroxide, high salinity and high osmolarity. Outer membrane integrity is closely related with bacterial survival under hostile environments, including extracellular and intracellular ones. Outer membrane is proven to be involved in resistance to intracellular hostile bactericidal factors (Roux et al., 2006; Teixeira-Gomes et al., 2000). The M5Δomp25 was more susceptible to polymyxin B, hydrogen peroxide, high sali-

nity and high osmolarity stress than their parent strain M5, again indicating the possible role of Omp25 in the *Burcella* intracellular survival. Greater decrease in log CFU was observed for M5Δomp25 at 8, 12 and 24 h p.i than M5 (Figure 3A), indicating that Omp25 are important for *Brucella* survival in macrophage. M5 and M5Δomp25 showed identical log cfu at 4 h p.i., suggesting that Omp25 is not essential for early survival in macrophage. Consistent with reduced survival in macrophage, M5Δomp25 showed decreased survival in Balb/c mice (Figure 3B).

To further assess the role of Omp25 in the immunological protection in the attenuated vaccine strain M5, the ability of M5Δomp25 to protect against *B. melitensis* virulent challenges was determined. M5 conferred significant protection against *B. melitensis* 16M virulent challenges in comparison to the unvaccinated control. However, the M5Δomp25 did not exhibit protection. Data from previously study showed that *B. melitensis* 16MΔomp25 mutant provided protection against virulent *B. melitensis* challenge at levels equal to strain Rev. 1 vaccine (Edmonds et al., 2002b), implying that omp25 is not the only protective antigen of *Brucella*. Results obtained here indicated that Omp25 is essential for the protection of M5 against *B. melitensis* 16M challenge in the mouse model.

The antibodies response and the cytokine profiles were also evaluated for M5Δomp25. Mice infected with M5 and M5Δomp25 could produce *Brucella*-specific antibodies. However, throughout the 56-day study, mice infected with the M5Δomp25 showed lower antibody titers than M5-infected mice. As is the case for many intracellular pathogens, the cell-mediated immunity elicited by vaccines plays critical roles in immunological protection against virulent strain infection (Eze et al., 2000; He et al., 2001). IFN-γ, a Th1 cytokine involved in pathogen clearance from activated macrophages, has been well defined as a critical factor in the control of *Brucellae* infections in mice via enhanced activation of macrophages and intracellular killing (Young, 1983; Zhong et al., 2009). Splenocytes from M5 immunized mice produced significantly higher level of IFN-γ in response to stimulation with heat-killed *Brucella* than that from non-immunized ones. IFN-γ production of stimulated splenocytes was detected at 7 days and peaked at 42 days post immunization. However, the levels of IFN-γ in stimulated spleen cells from mice immunized with M5Δomp25 exhibited a significant reduction when compared with M5. IL-10, a Th2 cytokine, is generally considered as an anti-inflammatory molecule, reducing negative effects caused by pro-inflammatory cytokines (Fernandes and Baldwin, 1995). Inhibition of IL-10 activity results in improved clearance of *Brucellae* from the spleens of infected mice (Fernandes and Baldwin, 1995). IL-10 production showed a pattern different from that of IFN-γ. IL-10 detected in spleen cells of M5Δomp25 and M5-infected mice. However, levels of IL-10 from M5Δomp25 infected mice were lower than from M5 infected ones at all the tested time points, especially at the late stage of infection (42 and 56 days post infection).

Antigen specific lymphoproliferation and production of IFN-γ reflect responses of sensitized T cells that should augment defense against *Brucella*. On the other hand, elicitation of IL-10 production in cells from immunized mice may counterbalance this effect [34, 35]. A number of studies have demonstrated antagonistic roles of these two cytokines in murine brucellosis (Young, 1983; Zhong et al., 2009). Although with lower production levels, M5Δomp25 induced the two cytokines as M5 did. Therefore, it is possible that the induction of IL-10 production plays a role in the survival of brucellae and may also reduce the immunogenicity of live attenuated vaccine by inhibiting robust development of a Th1-type response. A vaccine that selectively induced cells to make IFN-γ or failed to induce production of IL-10 might be more protective.

In summary, data from the present study indicated that Omp25 play important roles in both the virulence and immunological protection of the live attenuated vaccine strain M5. The *omp25* mutant showed a rapidly clearance from macrophages and mice. Furthermore, the mutant could not provide protection against virulent *B. melitensis* and elicited low levels of IgG and cytokines (IFN-γ and IL-10) when compared with M5. The absence of immunological protection, lower antibody titers, and shortened persistence of cell-mediated immune responses in mice infected with M5Δomp25 probably resulted from the more rapid clearance from spleen than their parent strain M5. Another possible reason is that Omp25 is an important antigen, which could induce and/or regulate immune response in mice. The results of the present study also implied that for an efficient live attenuated vaccine strain of *Brucella*, the strain should have enough survival capability in the hosts and induce high levels of cell mediated immunity. This finding is helpful for selection and evaluation of new live vaccine strains in the future with rational design.

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